EFFECT OF CHELATING AGENTS AND PHOSPHORAMIDON ON THE L-LEUCINE TRANSPORT SYSTEM IN MICROVILLAR VESICLES FROM PIG KIDNEY

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1. Introduction

The kidney proximal tubule is the site of transport of amino acids as well as other solutes present in the glomerular filtrate. The microvillar membrane that lines the tubule is enzymically complex, particularly in regard to the number and diversity of the peptidases it contains (reviewed in [1]). Several of these enzymes have been purified and characterized and some of them are known to be among the major proteins of the membrane. Aminopeptidase M (EC 3.4.11.2), dipeptidylpeptidase IV (EC 3.4.21) and neutral endopeptidase (EC 3.4.24) together contribute ~17% of the microvillar membrane protein. In contrast to these enzymes, the proteins concerned with amino acid transport have not yet been characterized. This is the more surprising since one would expect that a major function, such as amino acid transport, might be subserved by some of the major proteins of the membrane. Indeed, the existence of a group of microvillar peptidases, with no obvious physiological function, has prompted the speculation that they might play some role in amino acid or peptide transport [2].

Microvillar membrane vesicles were first used to study transport in [3] and this type of preparation is now widely used (reviewed in [4]). The transport by kidney microvillar vesicles of proline [5,6], alanine [7], cystine, arginine and other dibasic amino acids [8,9] have been reported. The uptake of proline and alanine is Na^+-dependent, manifested by an 'overshoot' of amino acid accumulation, subsiding as the Na^+ gradient dissipates towards equilibrium. This paper shows that the uptake of L-leucine proceeds by a similar process and that it may be inhibited by several reagents capable of interacting with divalent metals.

2. Experimental

Pig kidneys, generously given by Asda Farmstores Ltd., Lofthousegate, W. Yorkshire, were obtained within 5 min of death and immediately cooled on ice. Phosphoramidon (*N*-(α-L-rhamnopyranosyloxy-hydroxyphosphinyl-L-leucyl-L-tryptophan) [10] was a gift from Professor H. Umezawa, Institute of Microbial Chemistry, Kamiosaki, Shinagawa-Ku, Tokyo. L-[4,5-^{3}H]Leucine (TRK 170), 40–60 Ci/mmol and D-[1-^{3}H]glucose (TRK 285) 3–10 Ci/mmol, were from the Radiochemical Centre, Amersham, Bucks., HP7 0LL.

Microvillar vesicles were prepared from 20–30 g fresh pig kidney cortex [11]. The vesicle pellet was resuspended in 100 mM mannitol, 20 mM Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 7.4) to give 2.5 mg protein/ml. This suspension was incubated at 37°C for 30 min with or without inhibitors, then kept at 22°C until used. Transport of leucine and glucose was studied in an apparatus similar to that described in [12]. Two droplets, 40 μl vesicles (100 μg protein) and 10 μl containing 5 μCi L-[^{3}H]leucine (or D-[^{3}H]glucose), 1 mM L-leucine (or D-glucose), 0.5 M NaCl, 100 mM mannitol, 20 mM Tris/Hepes (pH 7.4), were pipetted separately into a 6.5 ml polystyrene tube and the two droplets fused by starting the shaker at ~100 Hz. The reaction was terminated by adding 2.5 ml 0.9% (w/v) NaCl, 1 mM

Tris/Hepes (pH 7.4) at 0°C. The contents were immediately filtered by suction (250 mm Hg) using a 0.45 μm pore cellulose membrane filter (Millipore HAWP, Millipore (UK) Ltd, Abbey Road, London NW10 7SP) pre-soaked in the wash solution, and washed with 2 × 2.5 ml of the cold 'stop' solution containing 200 μM L-leucine (or D-glucose). After drying, the filters were agitated in 1 ml 2-methoxyethanol, followed by the addition, 10 min later, of 10 ml scintillation fluid. Each experiment was done with quadruplicate values for each point and most experiments were repeated with 2 or 3 different vesicle preparations. A correction for nonspecific binding was made by subtracting zero-time radioactivity. The homogenates and vesicle preparations were assayed for aminopeptidase M and dipeptidylpeptidase IV by the methods in [13]. Protein was determined by A_{280} in the presence of 2% (w/v) sodium dodecylsulphate. The enrichment values were 10–16-fold compared with the homogenate.

3. Results

At 22°C the transport of L-leucine rose to a peak at 3 min, falling to ~40% of this value at 10 min and reaching an equilibrium value by 60 min. In most experiments an interval of 3 min was used, since this minimized errors. In the kinetic experiments, however, transport at 1 min was measured. This is only an approximation of the true initial rate of uptake since linearity is probably limited to the first few seconds [13]. In experiments showing a dependence on osmolarity, equilibrium values at 60 min were determined. The time course over the first 3 min is shown in fig.1. The dependency on a Na^+ gradient can also be seen: here the vesicles had been preincubated in 100 mM NaCl (i.e., at the initial gradient concentration), but the same effect was achieved by replacement of NaCl in the substrate droplet with choline chloride or by preincubating the vesicles with 5 μg gramicidin/mg protein. When the osmolarity of the medium was changed by varying the concentration of mannitol from 0.2–0.5 M the plot of L-leucine uptake (at 60 min) against $[\text{mannitol}]^{-1}$ was linear and extrapolated through zero. This result shows that the vesicles are osmotically active and that the phenomenon observed is transport of the amino acid rather than binding to the membrane surface.

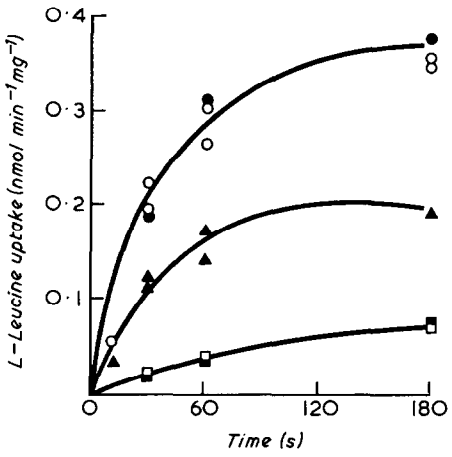

Fig.1. Time course of L-leucine uptake. Vesicles were preincubated for 30 min: (○) control vesicles (no additions); (●) 0.5 μM L-leucyl-L-tryptophan; (▲) 0.5 μM phosphoramidon; (■) 100 mM NaCl; (□) 100 mM NaCl, 0.5 μM phosphoramidon.

3.1. *Inhibition of transport by chelating agents*

Figure 2 shows the effects of EDTA, 1,10-phenanthroline, dithiothreitol and L-cysteine. All of these agents produced marked inhibition of L-leucine transport. The most effective was 1,10-phenanthroline with a K_i value (used here as the concentration giving 50%

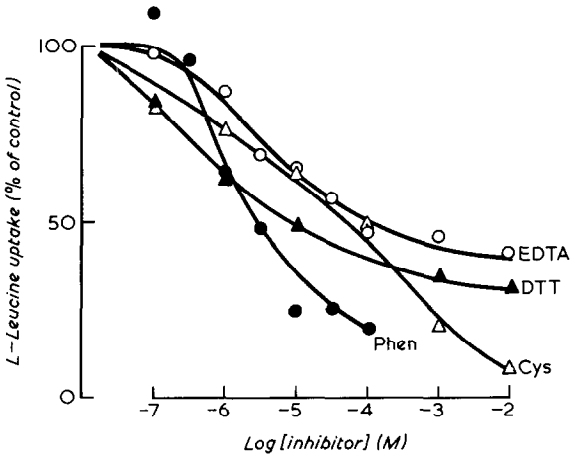

Fig.2. Inhibition of L-leucine uptake by chelating agents: (●) 1,10-phenanthroline (Phen); (○) EDTA; (▲) dithiothreitol (DTT); (△) L-cysteine (Cys). Results are means of 2 or 3 experiments.

inhibition of the gross uptake) of ~3 μM. EDTA was less inhibitory (K_i ~100 μM). Dithiothreitol also strongly inhibited (K_i ~10 μM), while the monothiol, L-cysteine, was rather less effective (K_i ~50 μM).

3.2. *Inhibition of transport by phosphoramidon*

Phosphoramidon at 0.5 μM inhibited the transport of L-leucine during the time course shown in fig.1. In this experiment the inhibition was ~50% and in 31 vesicle preparations this concentration has consistently inhibited (mean 57.7 ± 2.39, control = 100). Preincubation of the vesicles with the unsubstituted dipeptide, L-leucyl–L-tryptophan, (with or without P_i and L-rhamnose) at the same concentration, had no effect compared with control vesicles. Nevertheless in all experiments with phosphoramidon, L-leucyl–L-tryptophan at the same concentration was used as the control. The effect of phosphoramidon was only manifested on the Na^+-dependent component of the transport (fig.1).

The nature of the inhibition is shown in fig.3. In this experiment the uptake of L-leucine at 1 min was measured over 0.05–1.0 mM. The gross uptake values shown by the dashed lines (fig.3a) have been corrected by subtraction of the Na^+-independent component, which is linear with increasing concentration. These corrected values are plotted as reciprocals in fig.3b. The inhibition is non-competitive in type. The K_m for L-leucine transport, with an initial gradient of 100 mM NaCl, is 370 μM in this experiment. The binding of phosphoramidon to the membrane was freely reversible. This was shown in an experiment in which control and phosphoramidon-treated vesicles were washed (by suspending in inhibitor-free medium and centrifuging) such that the inhibitor would have been diluted > 1000-fold.

3.3. *Glucose transport*

The effects of some of the inhibitors on glucose transport differed from those observed with L-leucine. In two experiments phosphoramidon (0.5 μM), dithiothreitol (30 μM), EDTA (0.3 mM) had no effect. However, 1,10-phenanthroline (30 μM) and phlorizin (1 mM) both inhibited strongly (>95%).

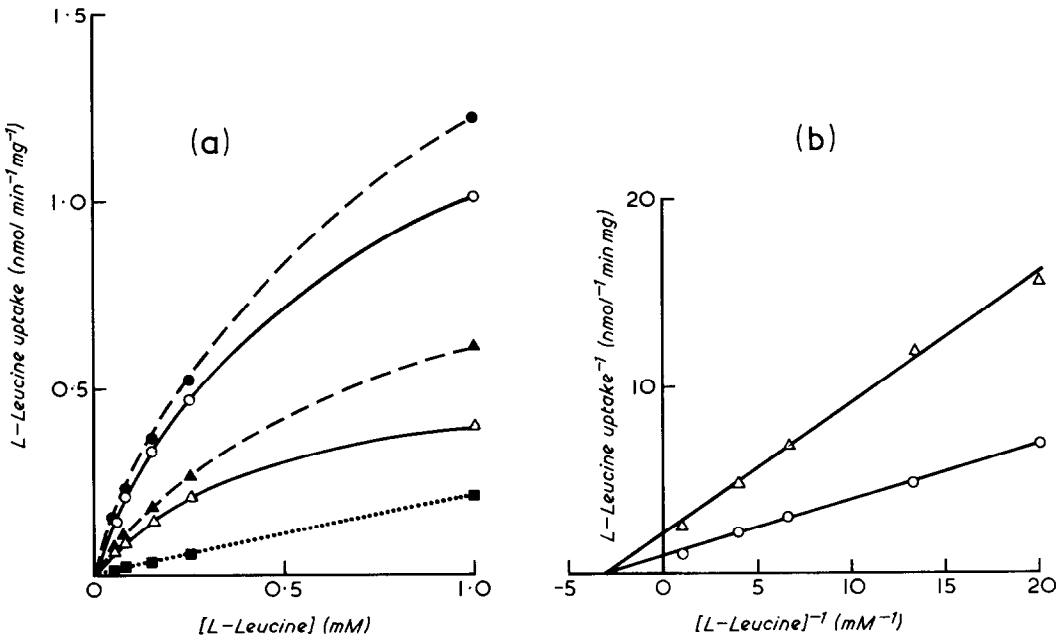

Fig.3. Kinetics of phosphoramidon inhibition of L-leucine uptake. Vesicles were preincubated with 0.5 μM L-leucyl-L-tryptophan (●,○), 0.5 μM phosphoramidon (▲,△) or 100 mM NaCl (■). The dotted line shows the Na-independent uptake, which in (a) has been subtracted from the gross uptake values (dashed lines) to reveal the Na-dependent uptake curves (continuous lines (○,△)) which are shown plotted as reciprocals in (b). Results are means of 2 experiments.

4. Discussion

Our results serve to define some of the properties of the L-leucine transport system in kidney microvilli. It is difficult to escape the conclusion that the transport protein contains a divalent metal ion: each of the four compounds, 1,10-phenanthroline, EDTA, cysteine and dithiothreitol, is a powerful chelating agent; the most potent of which, 1,10-phenanthroline, exhibits a high stability constant with Zn^{2+}, a metal known to be a constituent of several microvillar proteins [1]. The effect of phosphoramidon deserves further explanation. This N-substituted dipeptide was first isolated from *Streptomyces tanashiensis* and shown to be a specific inhibitor of thermolysin and related proteinases [10]. Work in this laboratory [14,15] had characterized neutral endopeptidase as a zinc metalloenzyme with properties, including specificity, similar to the microbial proteinases. This view was further supported by the demonstration that phosphoramidon was a potent competitive inhibitor (K_i 2 nM) of the microvillar endopeptidase [16]. No other microvillar peptidase has been shown to be susceptible to this inhibitor. The effects at low concentrations of all five inhibitors are not attributable to a general effect on membrane transport, as shown by the different results for glucose transport (though the strong inhibition by 1,10-phenanthroline is worthy of further study).

Our preliminary results may also encourage a speculation: that a single protein, hitherto identified as an endopeptidase, might mediate leucine transport. Both the peptidase and leucine transport are susceptible to the same group of inhibitors, one of which, phosphoramidon, may be regarded as possessing high specificity. There are, however, some quantitative discrepancies: the endopeptidase was generally less sensitive to the chelators though more susceptible to phosphoramidon. Moreover, the inhibition by phosphoramidon was noncompetitive for transport but competitive for hydrolysis. In addition, although the activity of the endopeptidase is partially dependent on ionic strength, it showed no specificity for Na^+ [15]. A model based on this speculation might postulate separate sites for peptide hydrolysis and amino acid transport with another site(s), for cations, exhibiting Na^+ specificity for the activation of transport though not for hydrolysis. We emphasise that such a model is highly speculative: it does, however, provide a basis for further experiments.

Acknowledgement

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